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13. ABSTRACT (Maximum 200 Words)

One of the hallmarks of cancer is uncontrolled cell growth and proliferation. In cells, a group of proteins called growth factor receptors are responsible for responding to the signals that trigger proliferation. When these receptors function abnormally due to genetic mutation, these can undergo uncontrolled proliferation and become cancerous. In the case of breast cancer, a specific group of growth factor receptors, the ErbB family, have been implicated in the formation of tumors

In order to prevent uncontrolled growth, normal cells tightly regulate the activity of the EGF receptors. Upon their activation, which initiates the signaling that triggers cell proliferation, the receptor is also programmed to be destroyed in order to stop growth factor-induced signaling. A defect in this destructive mechanism can result in abnormally active EGFR, which can lead to uncontrolled signaling, and thus tumor formation.

Recently, it has been shown that Hsp90 (heat shock protein 90) is able to bind EGFR, and prevent its destruction. The ability of Hsp90 to bind substrate proteins appears to be regulated by a small chemical modification termed reversible acetylation. Hsp90 appears to require deacetylation in order to properly interact with its substrates. This would suggest that the modulation of Hsp90 acetylation status will be important for EGFR-mediated cell proliferation.

Our lab has found that Hsp90 interacts with HDAC6 (histone deacetylase 6), which is a protein that acts to remove acetyl groups from proteins (deacetylation), and we hypothesize that this interaction plays a regulatory role in EGFR functioning. We aim to characterize the interaction between these proteins, and determine how this interaction effects EGFR signaling. In addition, we will examine the role of HDAC6 in EGFR-mediated breast cancer formation in a tissue culture model.

14. SUBJECT TERMS
HDAC6, EGFR, Hsp90, ubiquitination, reversible acetylation

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Table of Contents

INTRODUCTION:

An estimated 203,500 new invasive cases of breast cancer are expected to occur among women in the United States during 2002. Breast cancer is the leading cancer site among American women and is the leading cause of cancer deaths among women ages 40-59. Up to 30% of these tumors have mutated epidermal growth factor receptors (EGFR, also known as HER or ErbB1). EGFR has been shown to be significantly overexpressed and deregulated in breast cancer, and the related HER2 receptor (also known as ErbB2) has shown gene amplification in 15-30% of invasive ductal breast cancers. The deregulation and consequent uncontrolled growth factor signaling of these receptors correlate with increasing tumor size, spreading of tumors to the lymph nodes and high grade metastasis. Since the constitutive activity of ErbB receptors promotes breast cancer, it is important to determine exactly how the ErbB receptors are regulated. In addition, understanding the regulation of this receptor may prove helpful in the development of new drug targets to combat breast cancer, which appears to be of increasing importance in light of recent studies which suggest that current therapies may not be as effective as once predicted.

In this proposal, we have focused on defining a novel way of regulating EGFR signaling. It has been shown that Hsp90 acts to stabilize EGFR at the membrane, and consequently may promote proper EGFR signaling. It has also been shown that Hsp90 is an acetylated protein, and that its acetylation status regulates its interaction with other proteins that promotes EGFR signaling. Deacetylated Hsp90 is able to bind EGFR, and stabilize EGFR to allow for the initiation of signaling cascades. Our lab has shown that a cytoplasmic deacetylase, HDAC6, associates with Hsp90 and EGFR. We hypothesize that HDAC6 might regulate the acetylation status of Hsp90 and consequently be involved in the EGFR signaling cascade. The characterization of HDAC6 as an Hsp90 deacetylase would provide an essential drug target to combat the deregulated activity of EGFR in breast tumors. Confirmation of this idea would allow for targeted chemotherapeutics to be developed that specifically inhibit HDAC6 in order to block the EGFR signaling cascade, thus stopping uncontrolled growth and metastasis.

BODY: Research Accomplishments with Statement of Work

1. To characterize the functional significance of the HDAC6-Hsp90 interaction in EGFR signaling (Months 1-18)

a. Characterizing the interaction between HDAC6, Hsp90and EGFR. (Months 1- 8)

We have been able to show that HDAC6 interaction with Hsp90 increases upon stimulation with EGF (Data not shown). This interaction implies that Hsp90 effect on the stability of EGFR may be regulated by HDAC6. Additionally, Hsp90 is able to bind EGFR after ligand stimulation, thus implying the formation of a complex between these three proteins. We are currently exploring the possibility that these proteins interact *in vitro* in order to determine whether or not they bind directly or indirectly. Furthermore, we have found that the ubiquitin-binding domain of HDAC6 (BUZ Domain – Binder if Ubiquitin Zinc-finger domain) is required for HDAC6 binding to Hsp90 (Figure 1 – attached manuscript). This implies that (a) HDAC6 binding to Hsp90 is triggered by its association with ubiquitinated proteins (possibly EGFR), or (b) HDAC6 binds ubiquitinated Hsp90. These possibilities are currently being explored through the further examination of HDAC6 co-immunoprecipitations and examination of the ubiquitination status of the proteins involved. It does not appear that the ubiquitination status of Hsp90 modulates HDAC6 binding (data not shown).

b. Determining whether HDAC6 regulates Hsp90 acetylation and to examine the functional significance of such regulation. (Months 9-18)

We have found, using a combination of HDAC inhibitors and HDAC6 knock down cell lines that Hsp90 is deacetylated by HDAC6 (Figure 2 – attached manuscript). The use of TrichostatinA (TSA) to inhibit HDAC6 function results in a marked increase in the acetylation of Hsp90 (Figure 2). This effect, however, is not recapitulated in cells treated with nicotinamide, a NAD-dependent deacetylase inhibitor (Data not shown). Additionally, Hsp90 is heavily acetylated in A459 and 293T cells that stably express an siRNA for HDAC6 (Figure 2). HDAC6 protein levels are drastically decreased in these cells, and Hsp90 shows a marked increase in acetylation. We have shown that Hsp90 is unable to bind certain client proteins in these cells, and are currently in the process of creating MCF7 knock down lines in order to examine the effects of HDAC6 on ErbB2. It appears that these knock-down cells show a decrease in anchorage independent growth by soft-agar assay (Figure B)

Additionally, we have shown that, in another context, Hsp90 acetylation is important to its functionality. Hsp90 is known to mediate gluccocorticoid receptor activity, and we have shown through a transcriptional assay, that HDAC6-mediated deacetylation is required for this activity (Figure 3 – attached manuscript). Additonally, we have shown that this transcriptional decrease appears to be the result of the inability of GR to bind ligand (Figure 3). Furthermore, GR cannot translocate into the nucleus of HDAC6 knock-down cells after stimulation (Figure 3). The ligand binding activity of GR can be restored by the addition of deacetylated Hsp90 (Figure C). This proves that Hsp90 deacetylation is important for the proper functioning of Hsp90.

In addition to the above studies, we are currently in the process of mapping the acetylation sites on Hsp90. We have had no luck in determining the acetylation sites of Hsp90 using deletion mutants, and have now turned to a *in vitro* acetylation reaction using tritium to

radiolabel Hsp90. These samples will then be examined by mass spec in order to determine acetylation sites. Our lab has previously used this technique to map acetylation sites on p53 and MDM2, so we are confident that we can do the same for Hsp90.

2. To determine the role of HDAC6 in EGFR down-modulation (Months 19-30)

a. Examining the role of HDAC6 in the regulation of EGFR stability. (Months 19-24)

Using our HDAC6 knock-down lines we have determined that the half-life of EGFR after ligand stimulation is markedly decreased in cells stably expressing HDAC6 siRNA when compared to control cells (Figure D). These are the same cells which show a drastic increase in acetylated Hsp90, thereby causing us to form a hypothesis, whereby HDAC6 deacetylates Hsp90, allows it to interact properly with EGFR, and causes a stabilization of the receptor. Furthermore, we have also shown that stimulation of cells with EGF causes the deacetylation of Hsp90 by HDAC6 (Figure E). These data, coupled together imply that HDAC6-mediated deacetylation of Hsp90 controls the levels of EGFR in cells, and thus will regulate EGFR-mediated responses to growth factor signaling. We are continuing to explore these avenues at this time by checking to see if Hsp90 acetylation status can effect either the chemotactic response of cells to EGF or the activity of EGFR-triggered signal transduction pathways.

b. Examining whether HDAC6/Hsp90 regulates the ubiquitination of EGFR. (Months 24-28)

We are currently working on this aim, but have no data at this time.

c. Determining the role of HDAC6 in EGFR internalization and recycling. (Months 28-30)

We are currently exploring the possibility that HDAC6 effects endocytosis of receptors, but are only in the preliminary stages of this work. The increased degredation of EGFR in HDAC6 knockdown cells may be due to a deficiency in stability or a decrease in receptor recycling to the membrane. Further studies are needed to delineate between these two possibilities.

3. To asses the role of HDAC6 in breast cancer progression (Months 30-36)

a. Examining HDAC6 and EGFR-dependent proliferation in primary murine mammary epithelial cells (PMECs). (Months 30-33)

These experiments have yet to be undertaken. Additionally, we have acquired HDAC6 knock-out mice, and are currently redesigning our experiments in order to take advantage of these mice.

b. Determining the role of HDAC6 in modulating EGF-dependent cell motility. (Months 33-36)

We hypothesize that loss of HDAC6 will cause a decrease in chemotactic response to EGF. Preliminary results show that overexpression of HDAC6 causes an increase inresponse to growth factor stimulation in cells (Figure F). Interestingly, an Hsp90-binding deficient mutant ΔBUZ, is unable to cause this growth-factor-mediated increase in cell motility.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration of complex formation between HDAC6, Hsp90 and EGFR.
- Characterization of HDAC6 as an Hsp90 deacetylase.
- Demonstration that HDAC6 is essential for Hsp90 functionality and its proper binding to substrate proteins.
- Characterization of the binding of HDAC6 to Hsp90.
- Demonstration that HDAC6 mediates the stability of EGFR, possibly through its interaction with Hsp90.
- Demonstration that the acetylation status of Hsp90 is critically regulated by HDAC6 in response to EGF signaling.
- Demonstration that HDAC6 is required for motility increase in response to growth factor stimulation.
- Demonstration that loss of HDAC6 inhibits anchorage independent growth in tumor cells.

REPORTABLE OUTCOMES:

Published Articles:

Kawaguchi Y, Kovacs JJ, McLaurin A, Vance J, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell.* 2003 Dec 12;115(6):727-38.

Manuscripts in review:

The deacetylase HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Jeffrey J. Kovacs, Patrick J.M. Murphy, Stéphanie Gaillard, Xuan Zhao, June-Tai Wu, Christopher V. Nicchitta, Minoru Yoshida, David O. Toft, William B. Pratt, and Tso-Pang Yao (Molecualr Cell)

Submitted Articles:

Kovacs JJ, Hubbert C, Wu JT, Yao TP. A novel ubiquitin-binding zinc finger essential for HDAC6-induced chemotaxis links the acetylation and ubiquitination machinery. (Submitted)

Presentations:

Kovacs JJ. Regulation of molecular chaperones by reversible acetylation. Molecular Chaperones and the Heat Shock Response, Cold Spring Harbor, May 5th -9th, 2004.

CONCLUSION:

The EGFR is found to be upregulated in 15-30% of breast cancers. Deregulation of EGFR function leads to uncontrolled growth factor signaling, which correlates with increasing tumor size, spreading of tumors to the lymph nodes and high grade metastasis. Since the constitutive activity of EGF receptors promotes breast cancer, it is important to determine the mechanism by which EGF receptors are regulated. It has recently been shown that Hsp90 is necessary for stabilization of the EGFR protein itself, and that the interaction of Hsp90 with target proteins requires that Hsp90 be deacetylated. We have shown that one of the eleven characterized human histone deacetylases, HDAC6, specifically binds Hsp90. The hypothesis we wish to test is whether HDAC6-mediated reversible acetylation of Hsp90 plays a critical role in EGFR activity and whether HDAC6 contributes to the carcinogenic potential of de-regulated EGFR.

We have now shown that HDAC6 and Hsp90 interact in response to EGF-mediated signaling. This interaction led to the discovery of HDAC6 as an Hsp90 deacetylase. Interestingly, we have found that the deacetylation of Hsp90 is required for its proper functioning. Furthermore, it appears that HDAC6 plays a role in the general stability of EGFR in response to growth factor signaling, presumably through an Hsp90-dependent pathway. We are now continuing to characterize the important role of HDAC6 as an Hsp90 deacetylase, and are working to fully understand the functional consequences of Hsp90 deacetylation. In the next few months, we hope to apply these finding to experiments in the proposed cancer models to more fully understand the role of HDAC6 in tumor progression.

Due to the fact that EGFR signaling is a critical factor in breast cancer formation and metastasis, it is of great importance to understand the regulation of EGFR, and how changes in stability and internalization of this receptor contribute to carcinogenesis. The determination of HDAC6 as a factor that positively regulates EGFR signaling may provide an important drug target for future therapeutic strategies.

REFERENCES:

1. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP. HDAC6 is a microtubule-associated deacetylase. Nature. 2002 May 23;417 (6887): 455-8.
2. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance J, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell*. 2003 Dec 12;115(6):727-38.
3. Kovacs JJ, Hubbert C, Wu JT, Yao TP. A novel ubiquitin-binding zinc finger essential for HDAC6-induced chemotaxis links the acetylation and ubiquitination machinery. (Submitted).
4. The deacetylase HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Jeffrey J. Kovacs, Patrick J.M. Murphy, Stéphanie Gaillard, Xuan Zhao, June-Tai Wu, Christopher V. Nicchitta, Minoru Yoshida, David O. Toft, William B. Pratt, and Tso-Pang Yao (Molecualr Cell)

Figure A

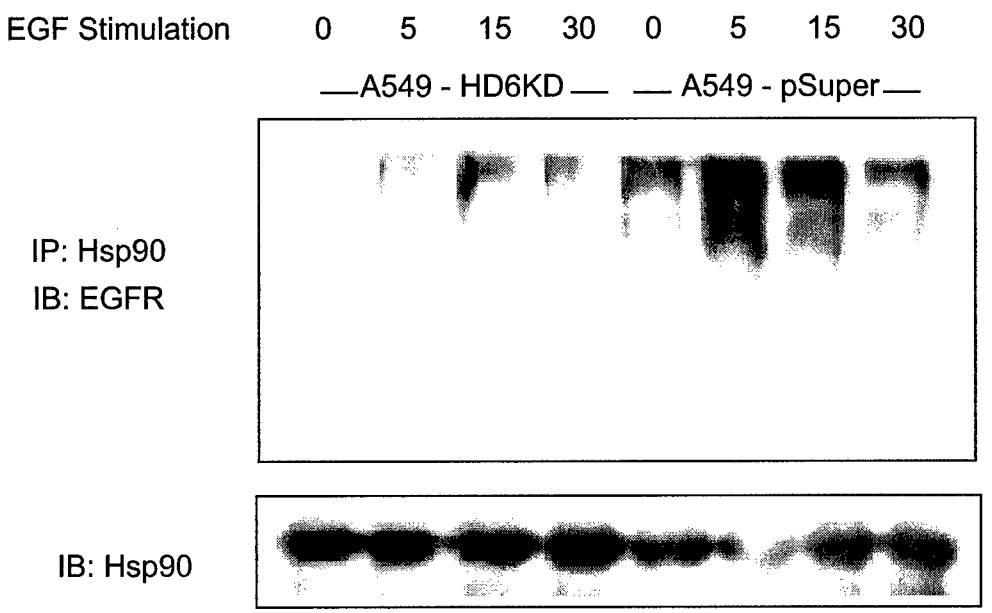
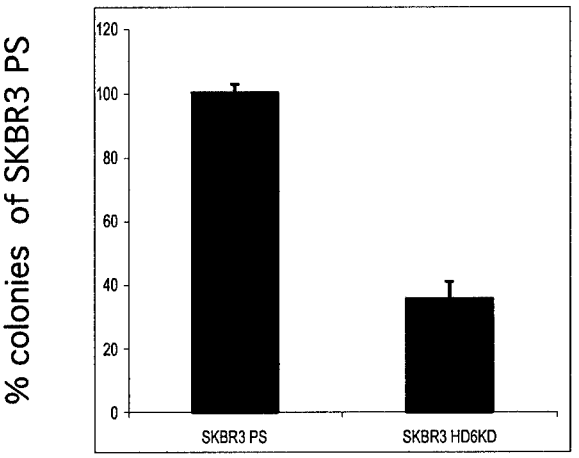


Figure A. A549 wt or HDAC6 knock-down cells were serum starved and then stimulated with EGF for the indicated time. Cell lysates were immunoprecipitated with Hsp90 and and then blotted for EGFR. High molecular weight species of EGFR were associated with Hsp90 in wt cells, but not in HDAC6 knock-down cells.

Figure B

A



B

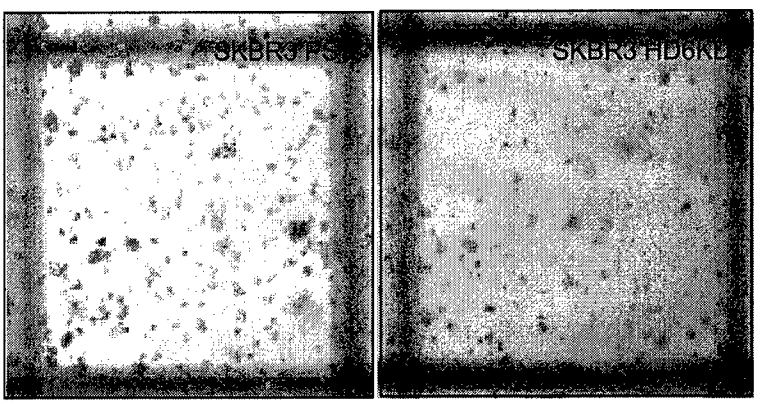


Figure B. HDAC6 is required for anchorage dependent growth of SKBR3 cells. A. Anchorage-independent growth of polyclonal SKBR3 cells expressing the siRNA against HDAC6 (HD6KD) or vector as negative control, was calculated from the average number of colonies ± standard deviation from six plates, expressed as the percentage of colonies observed in vector control cells. 50,000 cells per 35-mm-diameter plate cells were grown in 0.3% soft agar. The number of colonies >30 cells was scored after two weeks. Assays were done in triplicate and at least twice independentlystably. **B.** Representative image of soft agar.

Figure C

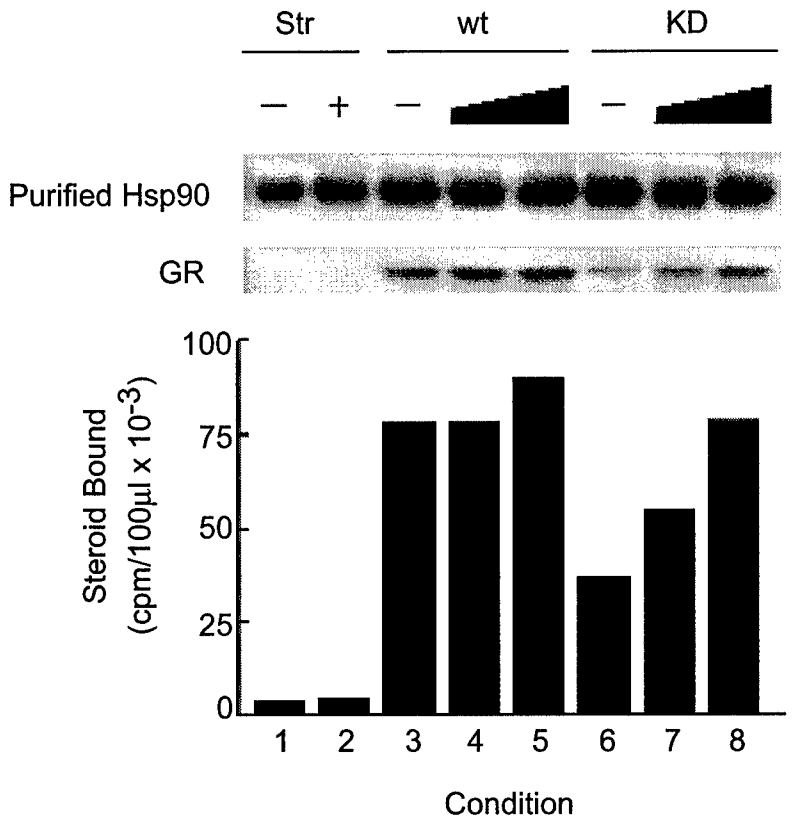
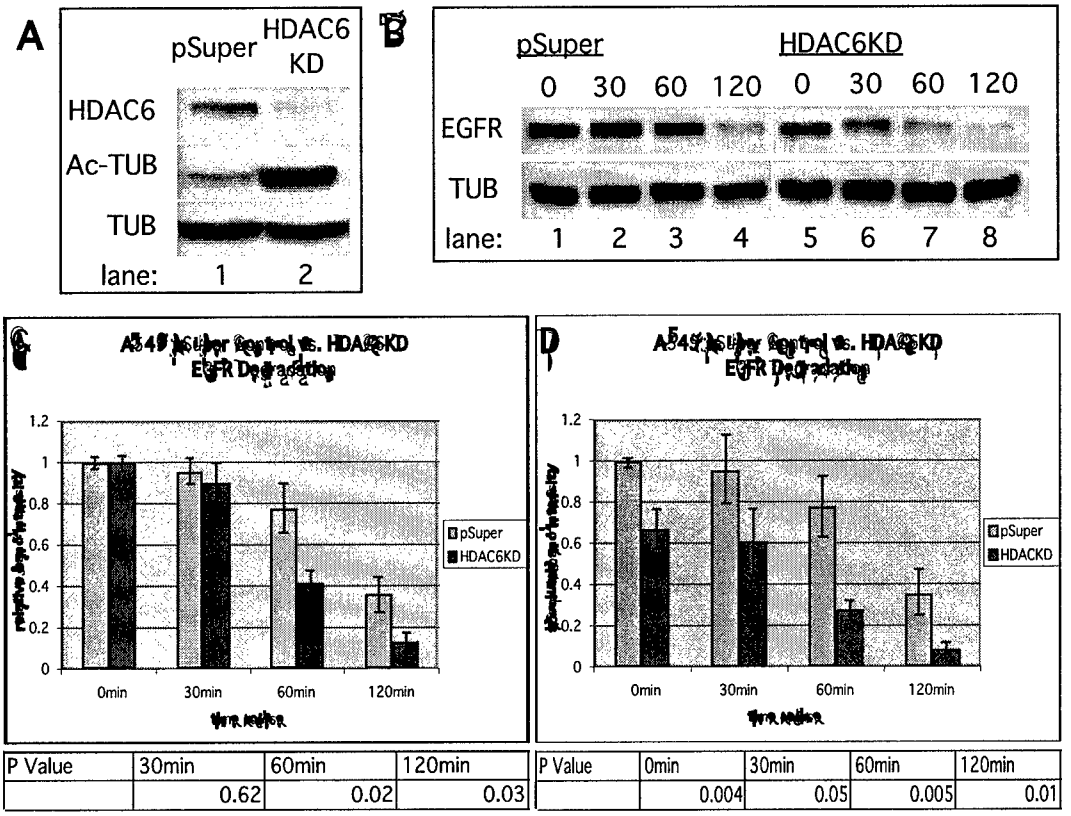


Figure C. Addition of purified Hsp90 to 293T-KDHD6 cell cytosol restores steroid binding activity and GR•Hsp90 heterocomplex formation to wild type levels. Stripped GR immune pellets were incubated with buffer (*Str*) or 293T cell cytosol stably transfected with either control (wt) or HDAC6 siRNA (KD) plasmid, in the presence of increasing concentrations of purified hsp90 and an ATP-regenerating system for 20 min at 30 °C. The immunopellets were washed and proteins were resolved by SDS gel electrophoresis and immunoblotted. Replicate immunopellets were washed and assayed for steroid binding activity. Conditions are: immunopellet incubated with buffer alone (*lane 1*) or 15 µg hsp90 (*lane 2*); immunopellet incubated with wt cytosol alone (*lane 3*), 7.5 µg hsp90 (*lane 4*), or 15 µg hsp90 (*lane 5*); immunopellet incubated with KD cytosol alone (*lane 6*), 7.5 µg hsp90 (*lane 7*), or 15 µg hsp90 (*lane 8*).

Figure D

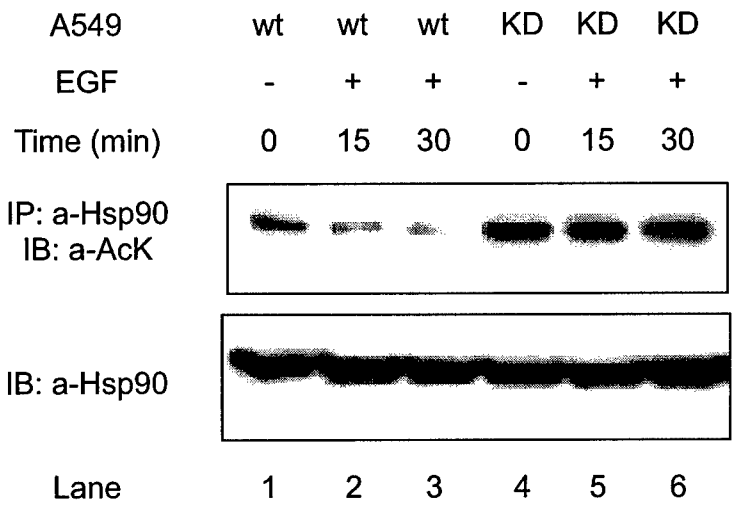


P Value	30min	60min	120min
	0.62	0.02	0.03

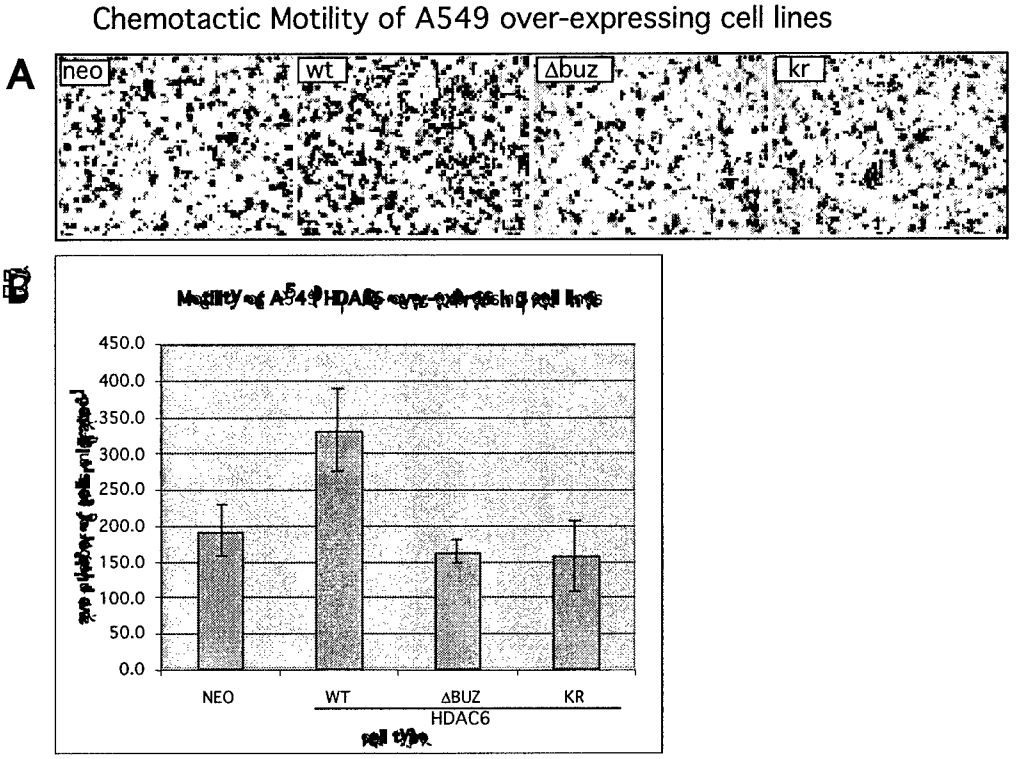
P Value	0min	30min	60min	120min
	0.004	0.05	0.005	0.01

Figure D. Knockdown of HDAC6 accelerates the rate of EGFR degradation. (A) A549 stable pSuper control and HDAC6 knockdown (HDAC6KD) cells were generated by retroviral infection. Western blot analysis show reduction in HDAC6 levels (top), a correlative increase in acetylated α-tubulin (middle) with α-tubulin as loading control (bottom). (B) A549 pSuper and HDAC6KD cells were serum starved, pre-treated for 30min with cyclohexamide, and stimulated with 100ng/ml EGFR. Western blots show total EGFR levels (top) and α-tubulin as loading control (bottom). (C) Graph of relative band intensity analysis of EGFR degradation Western blots comparing pSuper control and HDAC6KD levels for three independent experiments. (D) Graph of absolute band intensity analysis of EGFR degradation Western blots comparing pSuper control and HDAC6KD levels for three independent experiments.
Note that in the relative comparison there is an increase in degradation rate of EGFR in HDACKD cells. The absolute comparison further reveals a reduction in basal EGFR levels in HDAC6KD cells as well.

Figure E



A549 wt or KD cells were serum starved overnight in 0.2% FBS and then stimulated with 100 ng/ml EGF for the given amount of time. Cells were harvested and lysed according to previously described methods. IPs were performed using a-Hsp90 H1090 (Toft), and blots were immunoblotted with a-Acetyl Lysine (Yoshida). Note that basal levels of Hsp90 acetylation are higher in KD unstimulated KD lines (compare lanes 1 and 4). EGF stimulation triggers a deacetylation of Hsp90 (lanes 1-3) while this deacetylation does not occur in KD line (4-6).



P Value	NEO vs. WT	WT vs. ΔBUZ	WT vs. KR
	5.47769E-15	1.75374E-20	1.72062E-17
	ΔBUZ vs. KR	NEO vs. ΔBUZ	NEO vs. KR
	0.577	0.0002	0.002

Figure F . Overexpression of HDAC6 domain mutants affects chemotactic motility of A549 cells.
(A) A549 cells overexpressing control neomycin cassette (neo), wild-type HDAC6 (wt), ΔBUZ mutant HDAC6 (ΔBUZ), or KR mono-ubiquitin mutant HDAC6 (KR) were assayed for their chemotactic motility in a Transwell assay, which measured their ability to traverse an 8μm pore in a polycarbonate membrane in response to serum. Cells that traverse the membrane are visualized by coomassie blue stain. Representative fields are shown from one experiment. (B) Number of cells in the Transwell assay that exhibited chemotactic movement. The results represent an average of three independent experiments. Note that over-expression of wild-type HDAC6 increases the chemotatic response of A549 cells much like the affect in NIH3T3 cells, while over-expression of ΔBUZ mutant HDAC6, or KR mono-ubiquitin mutant HDAC6 does not. Note that these results indicate that as well as catalytic activity, HDAC6 requires both ubiquitin-binding activity and mono-ubiquitination for its ability to increase chemotaxis. The included table lists the statistical significance of this data by P Values. Note there is no statistical difference in motility between the ΔBUZ or KR mono-ubiquitin mutant HDAC6.

The deacetylase HDAC6 regulates Hsp90 acetylation and chaperone -dependent activation of glucocorticoid receptor

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Summary

The molecular chaperone heat shock protein 90 (Hsp90) and its accessory co-chaperones function by facilitating the structural maturation and complex assembly of client proteins, including steroid hormone receptors and selected kinases. By promoting the activity and stability of these signaling proteins, Hsp90 has emerged as a critical modulator in cell signaling. Here we present evidence that Hsp90 chaperone activity is regulated by reversible acetylation controlled by the deacetylase HDAC6. We show that HDAC6 functions as an Hsp90 deacetylase. Inactivation of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from an essential co-chaperone, p23, and a loss of chaperone activity. In HDAC6 deficient cells, Hsp90-dependent maturation of the glucocorticoid receptor (GR) failed, resulting in GR defective in ligand binding, nuclear translocation and transcriptional activation. Our results identify Hsp90 as a target of HDAC6 and suggest reversible acetylation as a novel mechanism that regulates Hsp90 chaperone complex activity.

Introduction

The heat shock protein Hsp90 and its co-factors form molecular chaperone complexes that facilitate the structural maturation of its substrates, termed client proteins. The Hsp90-assisted maturation of client proteins often leads to an enhanced activity and stability. Although initially recognized as a stress-induced protein, the realization that many Hsp90 targets are critical for normal and oncogenic signaling has identified Hsp90 as an important modulator in cell signaling and a promising target in cancer therapy (reviewed in (Neckers, 2002; Pratt and Toft, 2003)). The emerging significance of Hsp90 in both normal and oncogenic signaling underlines the fundamental importance to understand how Hsp90 activity is regulated.

The prominent examples of Hsp90 client proteins include steroid hormone receptors and kinases important for oncogenesis (Richter and Buchner, 2001). Among many client proteins, the Hsp90-dependent maturation of glucocorticoid receptor (GR), a member of the steroid hormone receptor, is best characterized. GR mediates biological effects of glucocorticoid by acting as a transcription factor (Giguere et al., 1986). Upon binding to glucocorticoid, GR becomes activated and translocates into the nucleus where it controls specific transcriptional programs. In the absence of its ligand, however, GR is inactive and resides in the cytoplasm where it associates with Hsp90 (Cadepond et al., 1991). Importantly, it has been shown that the association with Hsp90 is critical for GR to assume a ligand-binding competent conformation. Both *in vitro* and *in vivo* analyses demonstrate that Hsp90, in conjunction with a selected set of co-chaperone proteins, is required for GR to bind hormone with high affinity (reviewed in (Pratt and Toft, 2003)). The study of Hsp90-dependent GR maturation has provided mechanistic insight into the

basic steps of chaperone complex-client protein assembly and the important functions of co-chaperones (Dittmar et al., 1997). However, the critical question regarding whether and how Hsp90 itself is regulated in these processes is poorly understood.

Reversible protein acetylation has been characteristically linked to histone and chromatin-dependent processes. Recent studies, however, have revealed much broader arrays of biological processes that involve protein acetylation (reviewed in (Cohen and Yao, 2004)). For example, the characterization of the deacetylase HDAC6, a member of the histone deacetylase family, has implicated protein acetylation in the regulation of microtubules, growth factor-induced chemotaxis and the processing of misfolded protein aggregates (Haggarty et al., 2003; Hubbert et al., 2002; Kawaguchi et al., 2003; Matsuyama et al., 2002; Zhang et al., 2003). Consistent with these apparently non-genomic functions, HDAC6 is mainly localized to the cytoplasm (Hubbert et al., 2002; Verdel et al., 2000). These observations suggest reversible protein acetylation controlled by HDAC6 might have important regulatory roles in cytoplasmically-based processes. Identifying novel substrates for HDAC6 will be a critical step toward deciphering the general significance of protein acetylation in the cytoplasm.

In this report, we demonstrate that Hsp90 is a novel substrate of HDAC6 and its chaperone activity is regulated by acetylation. We show that HDAC6 and Hsp90 form a complex *in vivo* and that HDAC6 functions as an Hsp90 deacetylase. Inactivation of HDAC6 by pharmacological inhibition or by specific siRNA leads to hyperacetylation of Hsp90, the dissociation of an essential co-chaperone, p23, from Hsp90 and a loss of chaperone activity toward GR. We find that GR in HDAC6 deficient cells is defective in ligand binding, nuclear translocation and gene activation, revealing a failure of

chaperone-dependent maturation in the absence of functional HDAC6. Our results identify Hsp90/GR complex as a novel target of HDAC6 and suggest reversible acetylation as a critical mechanism in regulating Hsp90 molecular chaperone activity.

Results

HDAC6 associates with Hsp90 in vivo

To gain further insight into protein acetylation in the cytoplasm, we searched for cellular targets for HDAC6. Using an affinity trap approach, we identified Hsp90 as a prominent HDAC6 interacting partner by both mass spectrometry (data not shown) and direct immunoprecipitation, which shows that endogenous HDAC6 and Hsp90 can be abundantly and specifically co-immunoprecipitated from multiple cell lines (Figure 1A). To further characterize the HDAC6-Hsp90 interaction, we assessed whether mutations or pharmacological inhibitors that affect HDAC6 activity would influence its association with Hsp90. Full HDAC6 function requires both its deacetylase activity and a ubiquitin-binding activity, which is mediated by a unique zinc finger, termed the BUZ finger (Hubbert et al., 2002; Kawaguchi et al., 2003). We found that inactivation of HDAC6 either by mutations or by Trichostatin A (TSA) treatment led to the dissociation of HDAC6 from Hsp90 (Figure 1B, compare Lane 1 and 3 and 1C). Furthermore, an HDAC6 mutant lacking the ubiquitin-binding BUZ finger also fails to bind Hsp90 efficiently (Figure 1B, Lane 2). These data show that HDAC6-Hsp90 interaction is specific and it requires both deacetylase and ubiquitin-binding activities of HDAC6.

HDAC6 regulates Hsp90 acetylation

An interaction between HDAC6 and Hsp90 is significant as Hsp90 was recently reported to be an acetylated protein (Yu et al., 2002). We thus investigated the possibility that HDAC6 functions as an Hsp90 deacetylase. To this end, we first determined whether over-expression of HDAC6 can lead to Hsp90 deacetylation *in vivo*. As shown in Figure 2A (Lane 3), a basal level of Hsp90 acetylation can be detected in control A549 cell lines. Hsp90 acetylation levels, however, are markedly reduced in A549 cells that stably over-express wild type but not a catalytically inactive mutant HDAC6 (Figure 2A, compare Lanes 1 and 2). Conversely, in A549 cells stably expressing siRNA that reduces HDAC6 expression (HDAC6 knockdown (Kawaguchi et al., 2003)), Hsp90 acetylation levels are substantially increased (Figure 2B, compare lanes 1 and 4). These results show that HDAC6 can function as an Hsp90 deacetylase *in vivo*. Further supporting this conclusion, we found that TSA but not TrapoxinB (TPXb), a potent inhibitor for all HDAC members except HDAC6 (Furumai et al., 2001), also induces potent Hsp90 acetylation (Compare Figure 2B, Lane 1-3). Importantly, TSA treatment does not induce further Hsp90 acetylation in HDAC6 knockdown cells (Compare Figure 2B, Lane 4-6). Together, these data indicate that HDAC6 is the primary TSA-sensitive endogenous Hsp90 deacetylase.

Chaperone-dependent GR maturation is defective in HDAC6 deficient cells

We next determined if HDAC6-regulated Hsp90 acetylation is important for Hsp90 chaperone function. The requirement of Hsp90 chaperone activity for efficient ligand binding and the subsequent activation and nuclear translocation of the glucocorticoid receptor (GR) is the most well characterized function for Hsp90. To establish whether acetylation is important for Hsp90-dependent GR ligand binding,

cytosols prepared from control and HDAC6 knockdown 293T cells were incubated with radiolabeled dexamethasone, and steroid binding to the GR was determined. As expected, endogenous GR from control 293T cells binds ^{3}H-dexamethasone significantly (Figure 3A). However, a dramatic decrease in steroid ligand binding activity was observed in HDAC6 knockdown 293T cells (Figure 3A). Although comparable amounts of GR are present in cytosols from both cell types (Figure 3A, western blot), GR from the HDAC6 knockdown cells exhibited approximately a six-fold reduction in ligand binding activity, relative to control cells. This result demonstrates that GR produced in HDAC6 knockdown cells is defective in ligand binding activity, indicating an Hsp90 chaperone deficiency associated with Hsp90 hyperacetylation.

We further examined the functional status of GR by a transcriptional reporter assay. In control cells, endogenous GR potently induced a glucocorticoid-responsive element (GRE)-driven luciferase reporter following addition of the ligand, dexamethasone (Figure 3B, control). In contrast, dexamethasone treatment only weakly activated the GR reporter in HDAC6 knockdown 293T cells (Figure 3B, knockdown cells). Importantly, re-introduction of an siRNA-resistant plasmid expressing wild type HDAC6 (Kawaguchi et al., 2003) fully restored GR transcriptional activity in the HDAC6 knockdown cells (Figure 3B, +wt), whereas the catalytically-inactive or BUZ finger-deletion mutant HDAC6 were ineffective (Figure 3B, +cat-mut and +ΔBUZ), consistent with the observations that these mutants do not bind Hsp90 and cannot deacetylate Hsp90 efficiently (Figure 1B and 2A). The observed defect in transcriptional activity in HDAC6 knockdown cells is mirrored by the loss of ligand-induced nuclear accumulation of GR. In control A549 cells, GR becomes almost exclusively localized to

the nucleus within 30 minutes of ligand treatment (Figure 3D, ii). In contrast, GR remains in the cytoplasm in the substantial majority (~ 80%) of HDAC6 knockdown cells following ligand addition (Figure 3D, iv). Thus, Hsp90-dependent GR ligand binding, nuclear translocation and transcriptional activity are all defective in HDAC6 knockdown cells. Together, these results argue that HDAC6-mediated deacetylation is required for Hsp90 chaperone function to activate GR.

Hsp90 hyperacetylation is correlated with the dissociation of functional chaperone-GR complexes

The proper folding of GR by Hsp90 depends on the association of Hsp90 with a distinct set of co-chaperones into a chaperone complex (Reviewed in (Neckers, 2002; Pratt and Toft, 2003)). To identify the molecular basis for the regulation of Hsp90 function via deacetylation, we determined whether Hsp90 acetylation affects Hsp90/co-chaperone assembly. We focused on p23 (Johnson and Toft, 1994), a co-chaperone that stabilizes the Hsp90-GR complex and is critical for GR ligand binding activity *in vitro* and *in vivo* (Dittmar et al., 1997; Morishima et al., 2003). Co-immunoprecipitation assays demonstrate that Hsp90 associates with p23 in control cells (Figure 4A, Lane 1). However, Hsp90-p23 interactions are dramatically reduced in HDAC6 knockdown cells (Figure 4A, Lane 4). TSA treatment, which induces Hsp90 hyperacetylation, also disrupts Hsp90-p23 interactions (Figure 4A, lane 2). Conversely, TPXb treatment, which does not inhibit HDAC6 activity, has little effect (Figure 4A, Lane 3). These results support the hypothesis that Hsp90 acetylation leads to the dissociation of p23 from Hsp90. As p23 is known to stabilize the Hsp90-GR complex (Dittmar et al., 1997), we next examined whether acetylation affects Hsp90-GR complex formation. Indeed, the Hsp90 and GR

interaction is significantly reduced in HDAC6 knockdown cells or by treatment with TSA (Figure 4B, compare Lane 1, 2, and 3), providing a plausible mechanism for the observed GR defects. These results show that loss of HDAC6 activity leads to Hsp90 hyperacetylation, disassembly of the Hsp90 chaperone complex, and dissociation of the client protein GR.

Discussion

By promoting the activity and stability of many important signaling proteins, the molecular chaperone Hsp90 has recently emerged as a critical modulator in cell signaling and promising target in cancer therapy. Despite the apparent importance, the regulation of Hsp90 activity, chaperone complex formation and Hsp90-client protein interaction is not well understood. In this study, we identified HDAC6-regulated reversible acetylation as a novel mechanism that controls Hsp90 molecular chaperone function.

Hsp90 chaperone complexes stabilize client proteins and, in the case of GR, promote a conformation that allows efficient ligand binding and subsequent nuclear translocation and transcriptional activation. In this report, we demonstrate that GR produced in HDAC6 deficient model cell lines is defective in all three activities (Figure 3), strongly indicating a defect in Hsp90 chaperone function. Consistent with this conclusion, we found that Hsp90-p23 chaperone complex formation (Figure 4A) and chaperone-client (Hsp90-GR) association (Figure 4B) are both compromised in HDAC6 knockdown cells. The accumulation of hyperacetylated Hsp90 in HDAC6 deficient cells suggests that acetylation negatively regulates Hsp90 function by lowering Hsp90 affinity for the critical co-chaperone p23. Thus, stable complexes with client proteins, such as GR are not formed, resulting in a failure in client protein maturation (Figure 4). This model is

consistent with recent reports that several histone deacetylase inhibitors can induce Hsp90 acetylation and the dissociation of oncogenic client proteins from Hsp90 (Nimmanapalli et al., 2003; Xu et al., 2001). Our study now identifies HDAC6 as the primary Hsp90 deacetylase targeted by those inhibitors (Figure 2B). Surprisingly, however, GR remains stable in HDAC6 knockdown cells even though it no longer stably associates with Hsp90 (Figure 3C). This is in contrast to the effect of the Hsp90 inhibitor geldanamycin (GA), which induces GR degradation (Whitesell and Cook, 1996). These observations suggest that acetylation and geldanamycin, while both interfering with Hsp90 chaperone function towards GR, are not working through identical mechanisms. Understanding how acetylation and geldanamycin differentially affect Hsp90 function could provide important insight into the regulation of chaperone-dependent client protein maturation and degradation.

Although the exact mechanism by which acetylation regulates Hsp90 function remains to be established, and there may be additional chaperone components subject to reversible acetylation, our findings support the hypothesis that acetylation acts by modulating Hsp90 chaperone complex formation (Figure 4). Significantly, a dramatic remodeling of the Hsp90 chaperone complex has been observed upon normal cell transition to a tumor state (Kamal et al., 2003). This observation strongly suggests that the assembly of Hsp90 chaperone complexes is dynamically regulated and that its deregulation might play a key role for normal cells to undergo oncogenic transformation. We propose that reversible acetylation, controlled by HDAC6 and a yet to be identified molecular chaperone acetyltransferase, may be a physiological modulator that regulates Hsp90 chaperone complex remodeling and activity. Given the importance of Hsp90 for

many proteins vital to oncogenic signaling, the discovery of HDAC6 as a critical regulator of Hsp90 acetylation and function could have important therapeutic implications. The identification of the molecular chaperone Hsp90 as a new target for HDAC6 provides further support for broad functions of reversible protein acetylation and HDAC family members in important biological processes beyond histone and chromatin remodeling.

Materials and Methods

Cell lines – A549 and NIH-3T3 cell lines overexpressing HDAC6 wild type, ΔBUZ or catalytically inactive mutants were established through retroviral-mediated gene transfer. A549 and 293T cells stably expressing an siRNA for HDAC6 were established as described previously (Kawaguchi et al., 2003).

Antibodies – Rabbit polyclonal HDAC6 antibody DU227 was raised against a C-terminal HDAC6 peptide (DVKNAAHQNKFGEDMPHSH) followed by affinity purification and DU184 has been described previously (Hubbert et al., 2002). The production of antibodies for acetylated lysine (Komatsu et al., 2003), Hsp90 (H1090) and p23 (JJ3) has been described (Johnson and Toft, 1994). GR antibody was purchased from Cell Signaling. S-14 antibody recognizing the N-terminus of HDAC6 was purchased from Santa Cruz.

Immunoprecipitation and immunostaining – Cells were lysed as described previously (Hubbert et al., 2002). Hsp90 antibody was pre-incubated with rabbit-anti-mouse (Jackson Labs) and Protein-A Sepharose beads (Roche) for 10 minutes. The bead/antibody mix was added to 750μg of whole cell lysate and incubated at 4°C for 3 hours. Samples were spun-down, washed 4 times with 150mM NETN(Hubbert et al., 2002) and subjected to SDS-PAGE followed by immunoblotting analysis. Immuolocalization of GR and HDAC6 was performed as described previously(Hubbert et al., 2002).

Ligand Binding Assay - 293T cells stably transfected with either HDAC6 siRNA or control (pSuper) plasmid were lysed in 1.5 volumes of buffer (10 mM Hepes, pH 7.35, 1 mM EDTA, 20 mM Na_2MoO_4) and centrifuged at 100,000 x g. Aliquots (150 ul) of

cytosol were incubated overnight at 4°C with 100 nM [3H]dexamethasone plus or minus a 1,000-fold excess of non-radioactive dexamethasone. Free steroid was removed with dextran-coated charcoal, and steroid binding is expressed as cpm of [3H]dexamethasone/100 µl of cell cytosol, +/- SEM for three experiments with assays performed in triplicate.

Figure Legends

Figure 1 – HDAC6 associates with Hsp90. A. A431 (breast tumor) or A549 (small cell lung carcinoma) cell lysates were immunoprecipitated using α-HDAC6 antibody (DU227) or pre-immune serum (PI), and immunoblotted for Hsp90. Note that endogenous HDAC6 and Hsp90 co-immunoprecipitate while pre-immune serum does not pull down Hsp90. **B.** Cell lysates from NIH-3T3 cell lines stably overexpressing Flag-HDAC6, Flag-HDAC6-ΔBUZ (ubiquitin-binding deficient mutant), Flag-HDAC6-cat-mut (H216/611A, catalytically inactive mutant), or Neo vector control were immunoprecipitated using α-FLAG antibody and blotted with α-Hsp90. Hsp90 co-immunoprecipitates with wild-type HDAC6, but not with the ΔBUZ or H216/611A mutants. **C.** A549 cells were left untreated or subjected to a 4 hour treatment of trichostatin A (1μM). Cell lysates were then immunoprecipitated with α-HDAC6 antibody and then blotted for Hsp90. Note that treatment with TSA dissociates Hsp90 from HDAC6.

Figure 2 – HDAC6 regulates the deacetylation of Hsp90. A. Lysates from A549 cell lines stably overexpressing wild type (wt), catalytically inactive mutant HDAC6 (cat-mut) or Neo vector control were immunoprecipitated with α-Hsp90 and then immunoblotted with α-acetylated lysine antibody (α-AcK). Overexpression of wild type (wt) but not catalytically inactive mutant (cat-mut) HDAC6 caused a marked decrease in the levels of acetylated Hsp90. **B.** A549 cells stably expressing either pSuper control plasmid (wt) or HDAC6 siRNA (HDAC6 knockdown, KD) were left untreated, treated for 4 hours with TSA, or treated for 4 hours with TPXb (100nM). Cell lysates were then

immunoprecipitated with α-Hsp90 followed by immunoblotting with an α-AcK antibody. Note that both HDAC6 siRNA and TSA, but not TPXb treatment, cause a dramatic rise in the level of acetylated Hsp90 in control cells. TSA treatment did not further induce Hsp90 acetylation in HDAC6 knockdown cells.

Figure 3 – HDAC6 is required for GR ligand binding, translocation and transcriptional activity. A. Cytosols were prepared from control or HDAC6 knockdown 293T cells and subjected to ligand binding assay (see material and method). Steroid binding is expressed as cpm of [3H]dexamethasone/100 μl of cell cytosol +/- SEM for three experiments with assays performed in triplicate. Note Western blots for levels of HDAC6, Hsp90 and GR in the cytosol. **B.** Control or HDAC6 knockdown 293T cells were transiently transfected with an MMTV-GRE-luciferase reporter with or without expression plasmids of wild type, catalytically inactive (cat-mut) or ΔBUZ mutant HDAC6 as indicated. These plasmids contain silent mutations in the sequences targeted by siRNA for HDAC6. Relative luciferase activity was measured after 4 hour treatment with Dexamethasone and normalized to internal control (β-galactosidase). Note that the transfection of a wt-HDAC6, but not catalytically dead or ΔBUZ constructs, restored GR transcriptional activity in HDAC6 KD cells. **C.** The protein levels of GR and HDAC6 are determined by immunoblotting with antibodies against GR and HDAC6 respectively. GR levels are not affected by the status of HDAC6. **D.** Control and HDAC6 knockdown A549 cells were cultured in hormone free media for 24 hours, and then stimulated with dexamethasone for 30 minutes. The localization of GR was determined by immunostaining with an α-GR antibody. Immunofluorescence microscopy reveals

that GR shows a pan-cell staining before dexamethasone treatment in both cell types (i, iii). After dexamethasone treatment, GR efficiently translocates into the nucleus in control (ii), but not in HDAC6 KD cells (iv).

Figure 4 – The acetylation status of Hsp90 affects its interaction with the glucocorticoid receptor and the co-chaperone p23. A. Control A549 cells and HDAC6 siRNA knockdown cells (KD) were left untreated or treated for 4 hours with TSA or TPXb. Lysates were immunoprecipitated with α-Hsp90 antibody followed by immunoblotting with α-p23 antibody. Note that upon knockdown of HDAC6 (Lane 4) or treatment with TSA (Lane 2) but not TPXb (Lane 3), p23 no longer co-immunoprecipitates with Hsp90 efficiently. **B.** Lysates from control A549 cells or HDAC6 knockdown cells (KD) untreated or treated with TSA (4 hours) were immunoprecipitated with α-Hsp90 antibody followed by immunoblotting with α-GR antibody. Again, after treatment with TSA or knock-down of HDAC6, GR does not co-immunoprecipitate with Hsp90 efficiently.

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References:

Cadepond, F., Schweizer-Groyer, G., Segard-Maurel, I., Jibard, N., Hollenberg, S. M., Giguere, V., Evans, R. M., and Baulieu, E. E. (1991). Heat shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state. J Biol Chem *266*, 5834-5841.

Cohen, T., and Yao, T. P. (2004). AcK-knowledge reversible acetylation. Sci STKE *2004*, pe42.

Dittmar, K. D., Demady, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997). Folding of the glucocorticoid receptor by the heat shock protein (hsp) 90-based chaperone machinery. The role of p23 is to stabilize receptor.hsp90 heterocomplexes formed by hsp90.p60.hsp70. J Biol Chem *272*, 21213-21220.

Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. (2001). Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc Natl Acad Sci U S A *98*, 87-92.

Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645-652.

Haggarty, S. J., Koeller, K. M., Wong, J. C., Grozinger, C. M., and Schreiber, S. L. (2003). Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A *100*, 4389-4394.

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T. P. (2002). HDAC6 is a microtubule-associated deacetylase. Nature *417*, 455-458.

Johnson, J. L., and Toft, D. O. (1994). A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. J Biol Chem *269*, 24989-24993.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature *425*, 407-410.

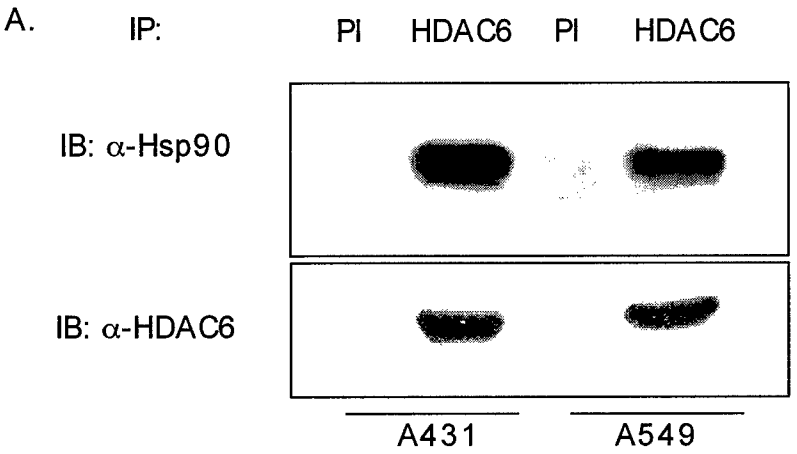
Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., and Yao, T. P. (2003). The Deacetylase HDAC6 Regulates Aggresome Formation and Cell Viability in Response to Misfolded Protein Stress. Cell *115*, 727-738.

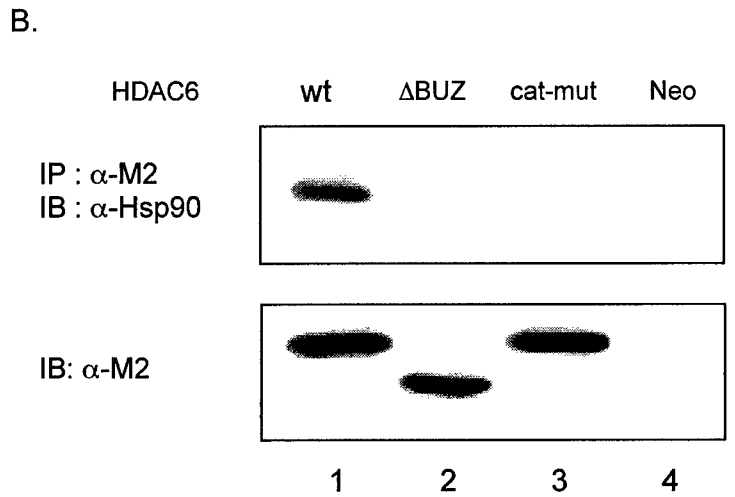
Komatsu, Y., Yukutake, Y., and Yoshida, M. (2003). Four different clones of mouse anti-acetyllysine monoclonal antibodies having different recognition properties share a common immunoglobulin framework structure. J Immunol Methods *272*, 161-175.

Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., *et al.* (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. Embo J *21*, 6820-6831.

Morishima, Y., Kanelakis, K. C., Murphy, P. J., Lowe, E. R., Jenkins, G. J., Osawa, Y., Sunahara, R. K., and Pratt, W. B. (2003). The hsp90 cochaperone p23 is the limiting component of the multiprotein hsp90/hsp70-based chaperone system in vivo

where it acts to stabilize the client protein: hsp90 complex. J Biol Chem *278*, 48754-48763.
Neckers, L. (2002). Hsp90 inhibitors as novel cancer chemotherapeutic agents. Trends Mol Med *8*, S55-61.
Nimmanapalli, R., Fuino, L., Bali, P., Gasparetto, M., Glozak, M., Tao, J., Moscinski, L., Smith, C., Wu, J., Jove, R., *et al.* (2003). Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res *63*, 5126-5135.
Pratt, W. B., and Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) *228*, 111-133.
Richter, K., and Buchner, J. (2001). Hsp90: chaperoning signal transduction. J Cell Physiol *188*, 281-290.
Verdel, A., Curtet, S., Brocard, M. P., Rousseaux, S., Lemercier, C., Yoshida, M., and Khochbin, S. (2000). Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr Biol *10*, 747-749.
Whitesell, L., and Cook, P. (1996). Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. Mol Endocrinol *10*, 705-712.
Xu, W., Mimnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. (2001). Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Biol Chem *276*, 3702-3708.
Yu, X., Guo, Z. S., Marcu, M. G., Neckers, L., Nguyen, D. M., Chen, G. A., and Schrump, D. S. (2002). Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. J Natl Cancer Inst *94*, 504-513.
Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S., and Matthias, P. (2003). HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. Embo J *22*, 1168-1179.





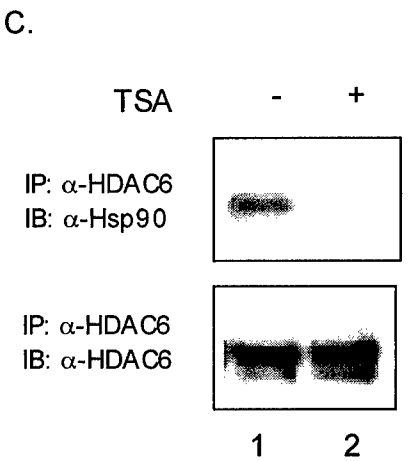


Figure 1 - Kovacs, et. al

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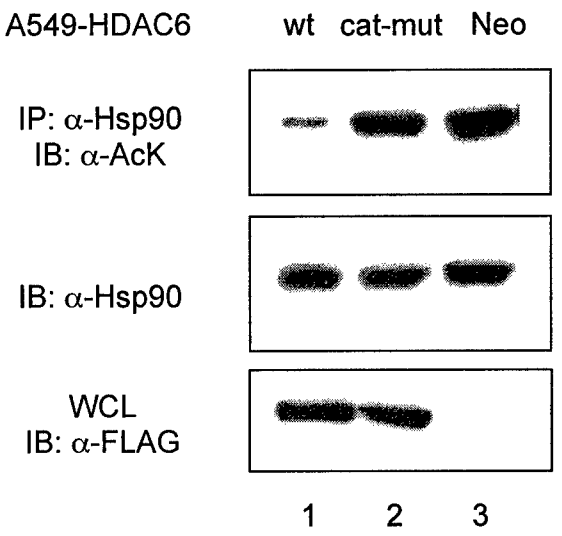

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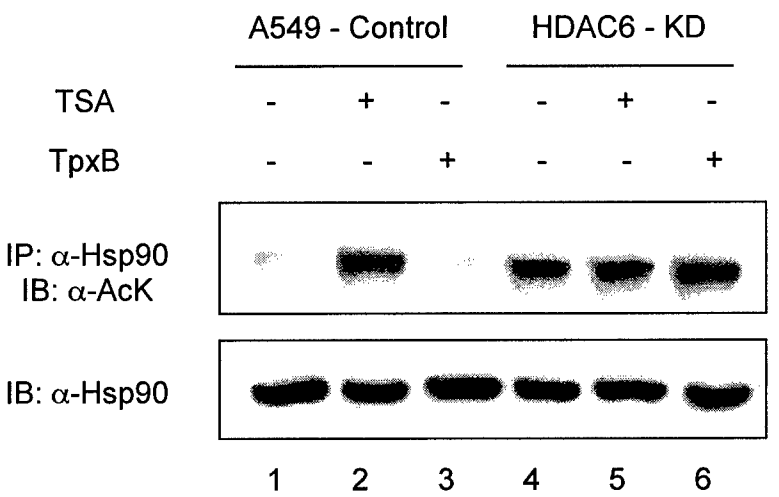

Figure 2 - Kovacs, et. al

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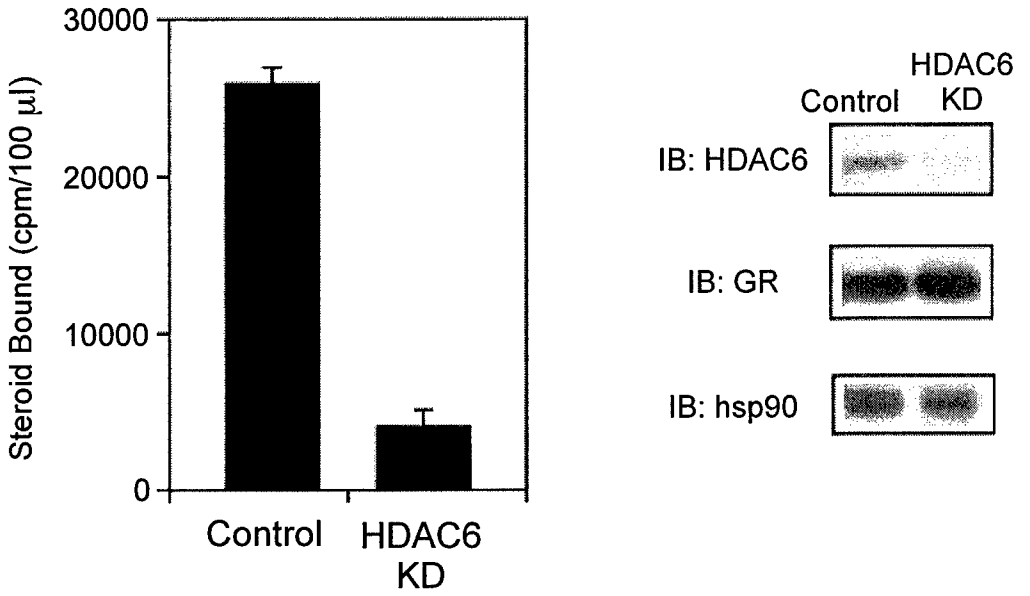

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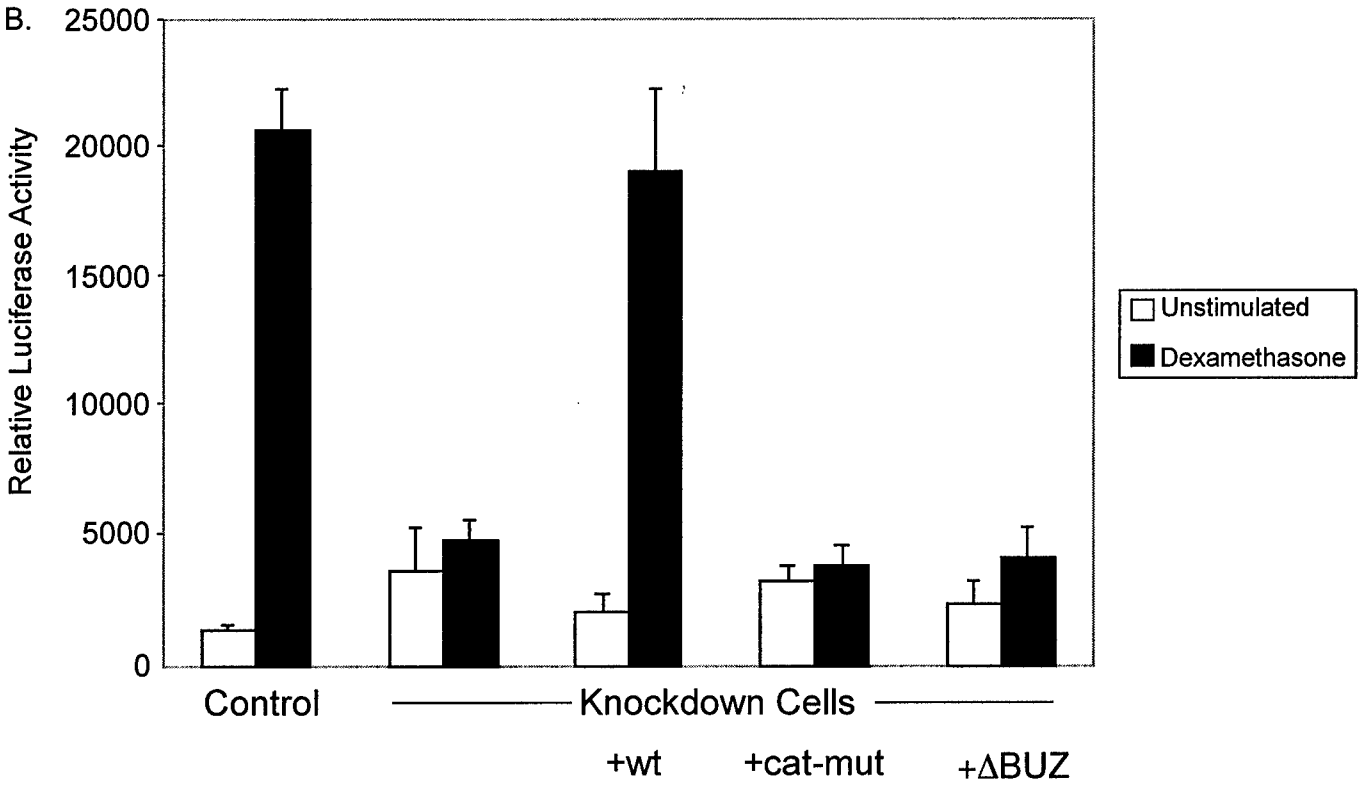

Figure 3 - Kovacs, et. al

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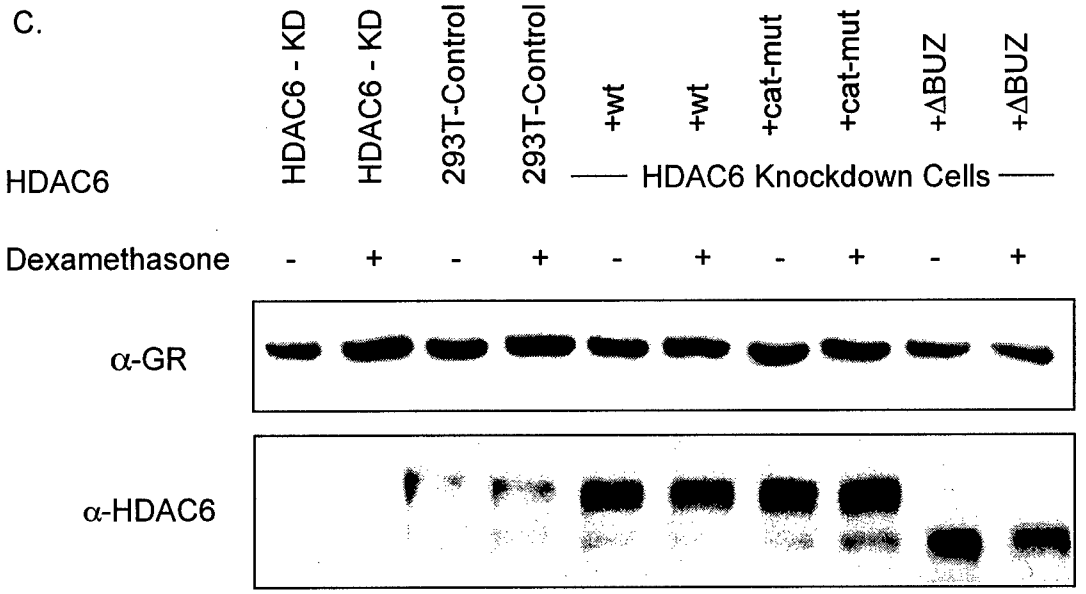

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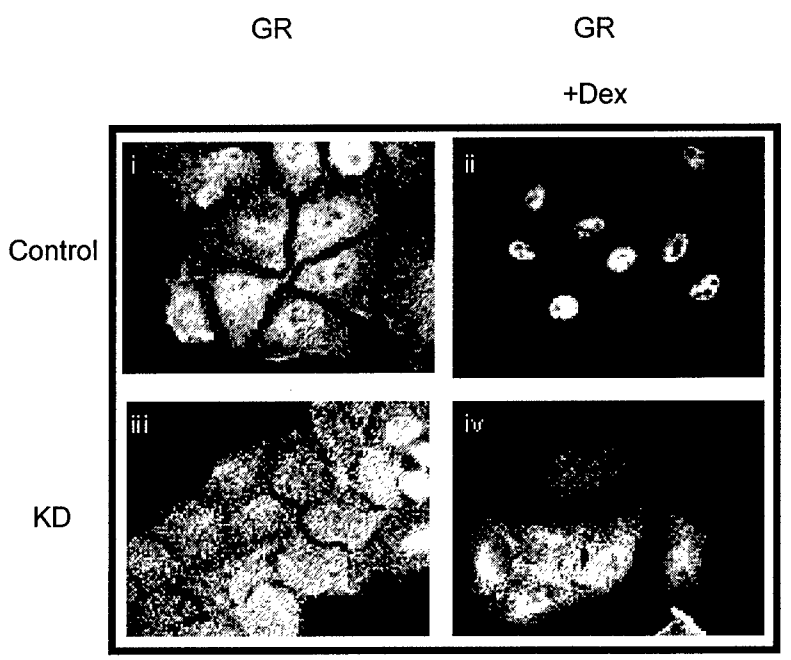

Figure 3 - Kovacs, et. al

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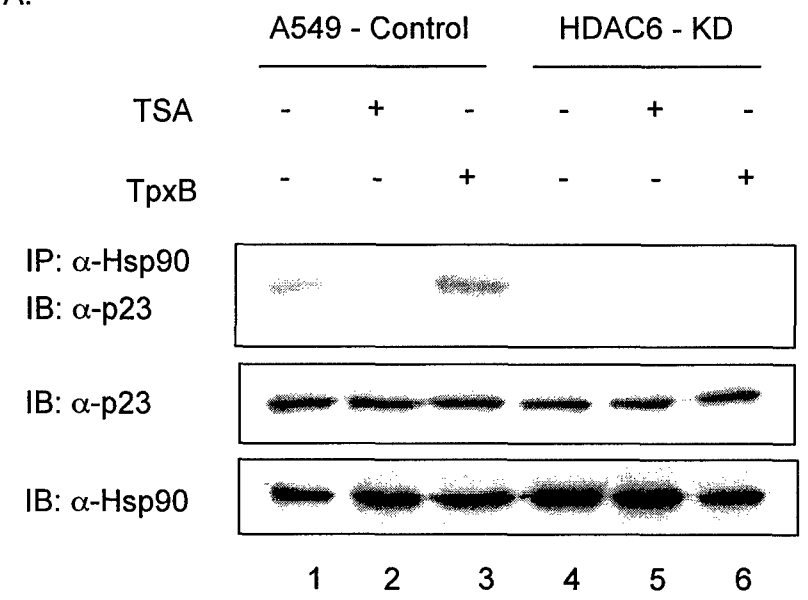

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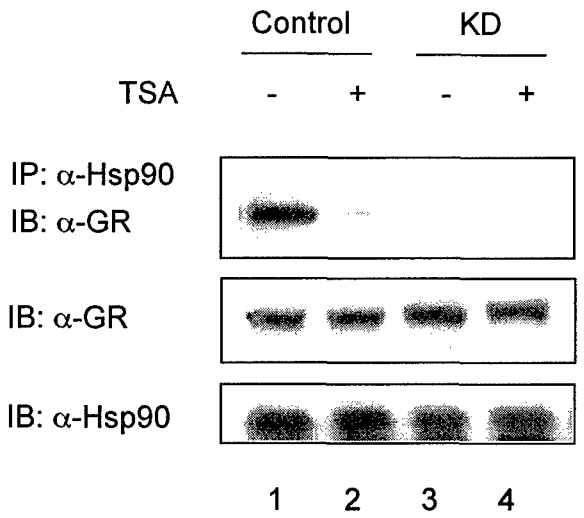

Figure 4 - Kovacs, et. al